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Distyly in *Psychotria serpens* (Rubiaceae) in the Ryukyu Islands, Japan

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Variation in several floral traits was examined to determine floral dimorphism in three populations of *Psychotria serpens* from Amami-Oshima and Okinawa islands, Japan. Two populations on Amami-Oshima exhibited long-styled and short-styled morphs that differed reciprocally from each other in stigma and anther heights, while a population on Okinawa was composed of long (L)-styled, short (S)-styled, and short homo (H)-styled morphs. In the L- and S-styled morphs, most flowers usually produced pollen grains stainable with aniline blue in lactophenol. Pollen grains from the S-styled morph were significantly larger than those from the L-styled morph, but no major difference was found between the two morphs in exine sculpturing. In the H-styled morphs, most flowers usually lacked pollen grains or did not produce stainable pollen grains. In some peculiar flowers, however, a few stamens that were exserted from the corolla tube rarely produced stainable pollen grains in the H-styled morph; the diameter of the pollen grains was in accordance with those of the S-styled morph. These observations suggest that *P. serpens* in the Ryukyu Islands is fundamentally distylous and sometimes includes short H-styled morphs within a population, the stamens of which are frequently abortive. Considering the nature of the peculiar flowers, the short H-styled morphs appear to be a consequence of unusual floral development.

Key words: breeding system, distyly, homostyly, pollen size, pollen stainability, *Psychotria serpens*, Rubiaceae

Distyly is a genetically controlled floral dimorphism in which plant populations are composed of two morphs, one with the stigma longer than the anthers (long-styled morph) and the other with anthers longer than the stigma (short-styled morph). The two morphs usually have an incompatiblity system that prevents self- and intramorph mating (Ganders 1979, Dulberger 1992, Barrett 1992, Naiki 2012). In addition, the two morphs often show morphological differences in corolla size, stigmatic papillae, pollen size, and exine sculpturing (Ganders 1979, Dulberger 1992). Distyly has repeatedly evolved in flowering plants, and has been reported in 26 angiosperm families (Naiki 2012). In the Rubiaceae, distyly has been found in more than 100 genera, and has been documented in species of *Psychotria* (Ganders 1979, Barrett 1992, Castro & Araujo 2004, Kondo *et al.* 2007, Sakai & Wright 2008, Naiki 2012).

Psychotria L. is a large genus of more than 1800 species widely distributed in tropical and subtropical regions of the world (Mabberley 2008, Davis *et al.* 2009). The species are usually understory shrubs, although trees, subshrubs, lianas, herbs, and epiphytes are also known (Nepokroeff *et al.* 1999, Sohmer & Davis 2007). The flowers of *Psychotria* are usually tubiform or campanulate and radially symmetrical. Within the genus, a distylous breeding system is one of the most important reproductive strategies. In Mesoamerica, 44 of 66 taxa are morphologically distylous while the remainder are monomorphic in floral morphology (Hamilton 1989). It was reported that 14 of 21 species co-occurring in Panama exhibited distyly while the others did not

(Sakai & Wright 2008). In Hawaii, all 11 species are morphologically distylous (Sohmer 1977) and functionally dioecious (Wagner *et al.* 1990, Sakai *et al.* 1995). In Japan, it has been reported that *P. boninensis* Nakai, endemic to the Bonin Islands, is distylous (Kondo *et al.* 2007), while *P. rubra* in the Ryukyu Islands and Taiwan is morphologically distylous but functionally dioecious (Watanabe *et al.* 2013). The breeding system of the remaining three species, *P. homalospermum* A. Gray, *P. serpens* L. and *P. manillensis* Bartl. ex DC, remains unclear.

Psychotria serpens is an evergreen epiphytic or terrestrial liana distributed widely from southwestern Honshu through the Ryukyu Islands in Japan to Taiwan, southeastern China, and Indochina (Yamazaki 1993). It grows at the edge or within broad-leaved forests. The cymose inflorescences, terminal on the branches, bear several to many actinomorphic, diurnally opening, somewhat tubiform flowers less than 5 mm long from late May to June. Each flower bears five (rarely six) stamens and a style with two stigmatic lobes, and lasts only a single day. The height of the stamens and style does not change after anthesis. White fruits mature in September to October. Although *P. serpens* was described as being distylous in Taiwan by Yang (1998), distyly has not been reported for the species in treatments of the flora of Japan, although it was noted that the anthers were exserted and the style was 2.5–3 mm long (Kitamura 1975, Yamazaki 1993). In this paper, we report distyly in *P. serpens* from Amami-Ohshima and Okinawa, Japan. We also report the occurrence of short homostylous flowers in one of the distylous populations.

Materials and Methods

Study sites

Field investigations were carried out on two populations of *Psychotria serpens* at Akasaki and Honcha Pass, Amami City, Amami-Oshima, Kagoshima Prefecture, and on one population on Nago-dake, Nago City, Okinawa, Okinawa Prefecture. The two islands belong to the Ryukyu Islands, Japan.

Measurement of floral traits

Floral traits were measured in 43 individuals from the Akasaki (AKA) population, 30 individuals from the Honcha Pass (HON) population, and 49 individuals from the Nago-dake (NAG) population. A few inflorescences with fully open flowers and flower buds were collected from each individual at random and preserved in 70% ethanol. After fixation, we measured one flower per individual. The measured parts were: (a) height from the base of the corolla tube to the apex of the highest anther (anther height), (b) height from the base of the corolla tube to the apex of the stigma (stigma height), and (c) distance from the base to the distal end of the corolla tube (corolla tube length) (Fig. 1).

Pollen stainability, pollen diameter, and pollen morphology

Pollen stainability with aniline blue in lactophenol of 200 to 270 pollen grains per flower was determined for 43 flowers from the AKA population, 30 flowers from the HON population, and 49 flowers from the NAG population (Appendix 1). Each flower was picked from a different individual.

For measurement of pollen size, anthers within a flower bud before anthesis were crushed in aniline blue in lactophenol. Subsequently, the polar diameter of 25 randomly selected pollen grains was measured using a light microscope. Exine sculpturing of pollen grains was also observed using a scanning electron microscope. In this case, anthers collected from a flower bud were dehydrated in an ethanol: t-butanol series. Dehydrated materials were freeze-dried using a JFD-300 freeze-drying device (JEOL), mounted onto scanning electron microscopy stubs with double-sided carbon tape, coated with gold using a JFC-1100E ion sputter (JEOL) and observed using a JSM-5600LV scanning electron microscope (JEOL).

Fruit set in open pollination

Fruiting under open pollination was determined for 11 individuals sampled from the NAG population in 2008, and fruit set (mature fruit per

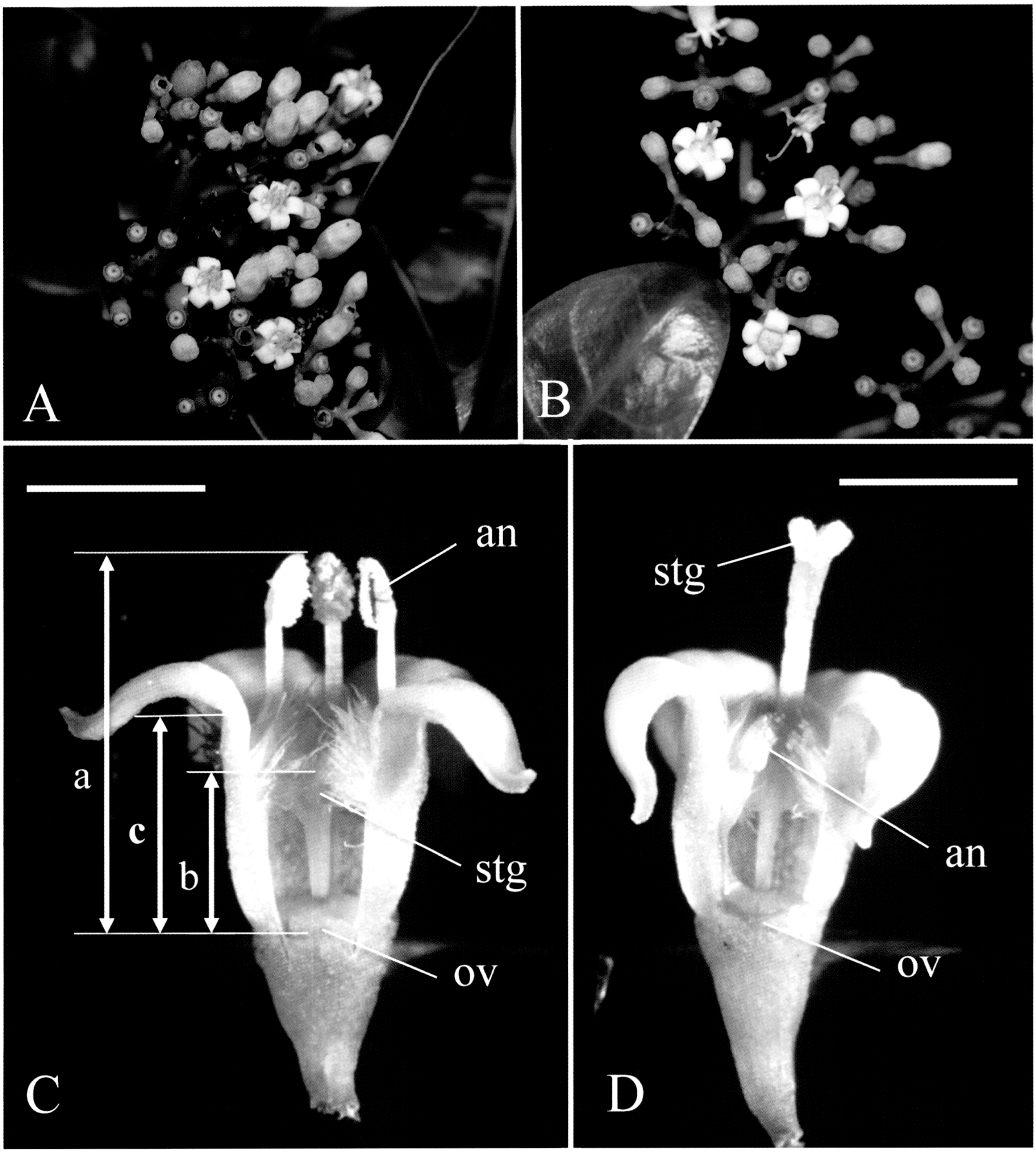

FIG. 1. Two different floral morphs in the Akasaki population of *Psychotria serpens* on Amami-Oshima Island. A, Inflorescence with short-styled flowers. B, Inflorescence with long-styled flowers. C, Short-styled flower; part of the corolla and two of the five stamens have been removed. D, Long-styled flower; part of the corolla and one of the five stamens have been removed. an, anther; stg, stigma; ov, ovary. a, anther height; b, stigma height; c, corolla tube length. Scale bar = 2.5 mm.

flower) of these individuals was quantified.

Statistical analyses

All statistical tests (Mann-Whitney U test, Steel-Dwass' multiple range test, Fischer's exact test, and Bonferroni adjustment procedure) were performed using Excel Statistics for Macintosh ver. 5.0 software (Esumi, Co., Ltd.).

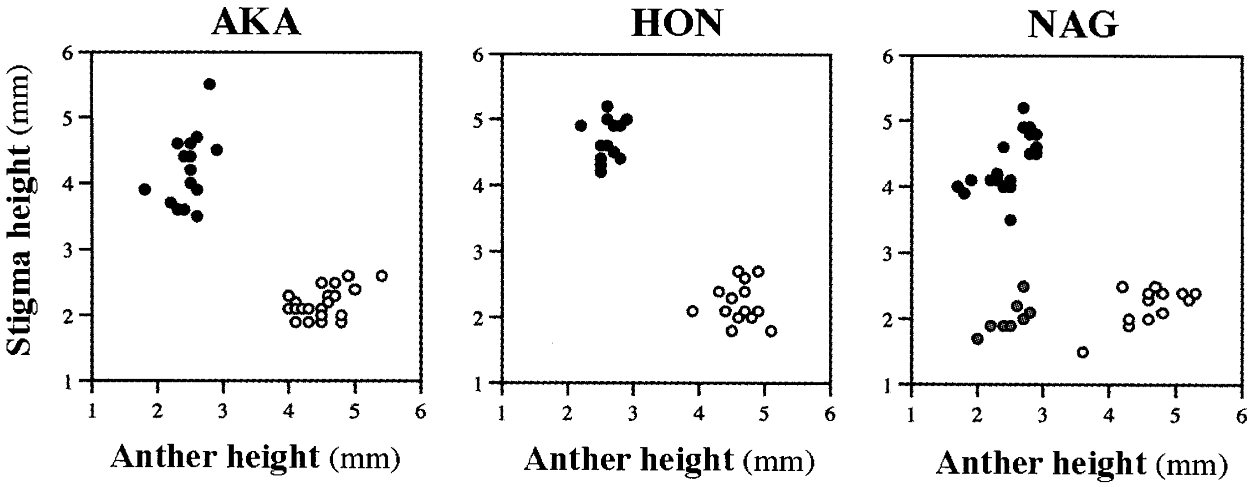

FIG. 2. Relationship between anther height and stigma height of one flower for each individual examined in three populations of *Psychotria serpens*. Closed dots, open dots, and gray dots denote L-styled, S-styled, and short H-styled morphs, respectively. AKA, Akasaki population; HON, Honcha Pass population; NAG, Nago-dake population.

TABLE 1. Measurements of floral traits, pollen stainability, and pollen size in L- and S-styled morphs in AKA and HON populations of *Psychotria serpens* on Amami-Oshima Island.

Population	L-styled	S-styled	Statistics
Character	Mean ± SD	Mean ± SD	(Mann Whitney's U-test)
Akasaki (AKA)			
Number of study flowers	19	24	
Corolla tube length (mm)	2.2 ± 0.31	2.4 ± 0.18	$0.05 < P < 0.01$
Anther height (mm)	2.5 ± 0.24	4.5 ± 0.38	$P < 0.001$
Stigma height (mm)	4.2 ± 0.51	2.2 ± 0.22	$P < 0.001$
Pollen stainability (%)	94.4 ± 6.7	90.9 ± 11.8	ns
Honcha Pass (HON)			
Number of study flowers	15	15	
Corolla tube length (mm)	2.5 ± 0.15	2.5 ± 0.26	ns
Anther height (mm)	2.6 ± 0.18	4.5 ± 0.54	$P < 0.001$
Stigma height (mm)	4.7 ± 0.31	2.2 ± 0.30	$P < 0.001$
Pollen stainability (%)	88.8 ± 18.6	85.6 ± 20.8	ns

ns: not significantly different between two floral morphs.

Results

Floral morphs in three populations

Morphological observations revealed that both the AKA and HON populations were composed of two distinct floral morphs: a short (S)-styled morph with long stamens, and a long (L)-styled morph with short stamens (Figs. 1 & 2). The two floral morphs have never been observed in the same individual; some individuals exclusively possess flowers of the L-styled morph and the others exclusively possess flowers of the S-styled morph. The number of flowers per inflorescence varies considerably among individuals and even among the inflorescences of the same individual. We could not find major differences in number of flowers per inflorescence between the two morphs.

In the S-styled flowers, stamens with a long filament were exserted from the corolla tube and the anthers were situated above the stigma, which was concealed by a number of obliquely oriented hairs on the corolla tube (Fig. 1C). In the L-styled

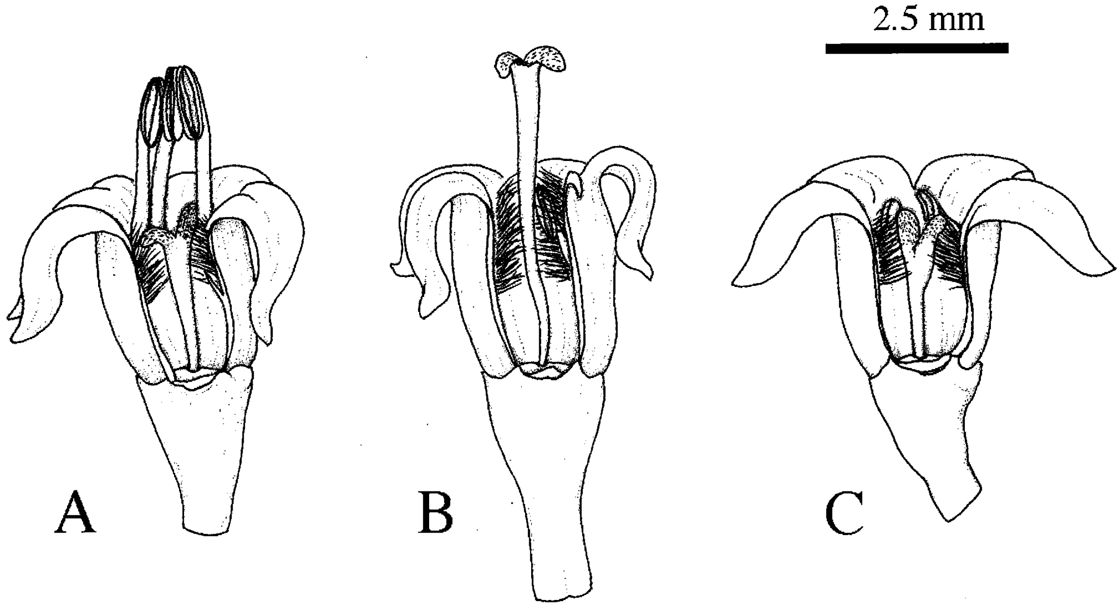

FIG. 3. Schematics of three flower morphs in the Nago-dake population of *Psychotria serpens* on Okinawa Island. A, S-styled morph; B, L-styled morph; C, Short H-styled morph. Scale bar = 2.5 mm.

TABLE 2. Measurements of floral traits, pollen stainability, and pollen size in L-, S-, and H-styled morphs in the NAG population of *Psychotria serpens* on Okinawa Island. Values sharing the same letters (a, b) indicate no significant difference among floral morphs at $P < 0.05$ by a Steel-Dwass's multiple range test.

Population	L-styled	S-styled	H-styled
Character	Mean ± SD	Mean ± SD	Mean ± SD
Nago-dake (NAG)			
Number of study flowers	24	16	9
Corolla tube length (mm)	2.5 ± 0.26 a	2.6 ± 0.35 a	2.2 ± 0.18 b
Anther height (mm)	2.5 ± 0.36 a	4.6 ± 0.46 b	2.5 ± 0.27 a
Stigma height (mm)	4.4 ± 0.44 a	2.2 ± 0.29 b	2.1 ± 0.28 b
Number of study flowers	16	16	3
Pollen stainability (%)	82.4 ± 31.8 a	95.4 ± 5.2 a	96.7 ± 4.7 a

flowers, the stigma was exserted from the corolla tube, and situated above the anthers, which were concealed by obliquely oriented hairs on the corolla tube (Fig. 1D). Mean values of corolla tube length, anther height, and stigma height in the two morphs are shown in Table 1. Stigma and anther heights differed significantly between the two morphs. The corolla tube was significantly longer in the S-styled morph than in the L-styled morph in the AKA population, but did not differ significantly in length between the two morphs in the HON population (Table 1).

In the NAG population, we found three floral morphs: long (L)-styled, short (S)-styled, and short homo (H)-styled morphs (Figs. 2 & 3). The L- and S-styled morphs were the same as those in the two populations on Amami-Oshima. Individuals with flowers of the short H-styled morph produced neither L-styled morph flowers nor S-styled morph flowers; they bore short H-styled morph flowers exclusively. Such flowers have the stigma positioned at the same height as the anthers and not exserted from the corolla tube (Fig. 3c). This morph was found in all nine individuals examined (Table 2). In three of the individuals with H-styled morphs, however, some inflorescences rarely had a few peculiar flowers that differed from the typical H-styled ones; one or two stamens were exserted from the corolla tube and the others were not (Table 2) and, as described below, their anthers produced stainable pollen grains. Mean values of corolla tube length, anther height, and stigma height determined for the three morphs are summarized in Table 2. In this population, corolla tube length was significantly longer in the S- and L-styled morphs than in the H-styled morph.

In all three populations, L- and S-styled flowers were not exactly reciprocal; i.e., the stigma height of one morph often differed statistically from the anther height of the opposite morph (Mann-Whitney U test, $P < 0.05$ for anther height of L-styled morph × stigma height of S-styled morph in AKA, HON, NAG; $P < 0.05$ for stigma height of L-styled morph × anther height of S-styled morph in AKA; $P = 0.37$ for stigma height of L-styled morph x anther height of S-styled morph in HON; $P = 0.09$ for stigma height of L-styled morph x anther height of S-styled morph in NAG).

Pollen stainability, pollen size, and exine sculpture in the three floral morphs

In the AKA and HON populations, the L- and S-styled morph flowers normally produced pollen grains that usually showed high pollen stainability (Fig. 4, Appendix 1). Between the two morphs, no significant difference was found in pollen stainability (Table 1).

In the NAG population, all individuals with the S-styled morph flowers normally produced pollen grains and showed high values for pollen stainability (Table 2). Most individuals with L-styled morph flowers also produced stainable pollen grains, but several of them completely lacked pollen grains in their stamens or produced nonstainable pollen grains (Table 2; see also Appendix 1). In contrast, individuals with short H-styled morph flowers usually lacked pollen grains or produced nonstainable pollen grains in their stamens (Appendix 1). However, three individuals with this morph occasionally bore a few peculiar flowers with one or two exserted stamens together with short stamens. The exserted stamens produced stainable pollen grains in their anthers and showed high pollen stainability comparable to those of the S-styled morph flowers (Table 2).

Pollen grains from all three morphs were spherical and tricolporate and their exine sculpturing was reticulate, having a net-like sculpturing (Fig. 5). We found no major differences among the morphs in exine sculpturing.

The diameter of pollen grains from the S-styled morph flowers was significantly greater than in the L-styled morph flowers in all populations examined (Fig. 6; see also Tables 1 & 2). The pollen size ratio of the S- to L-styled morphs was 1.31 in the AKA population, 1.35 in the HON population, and 1.38 in the NAG population. Pollen grains from the H-styled morph, which were

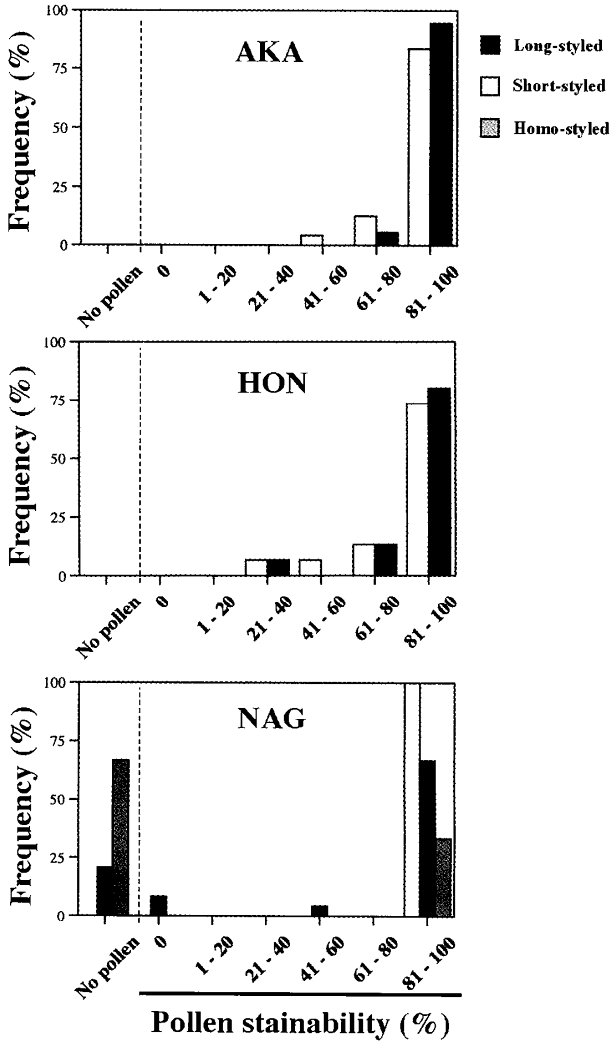

FIG. 4. Frequency distribution of pollen stainability in L-, S- and H-styled morphs in three populations of *Psychotria serpens*. AKA, Akasaki population; HON, Honcha Pass population; NAG, Nago-dake population.

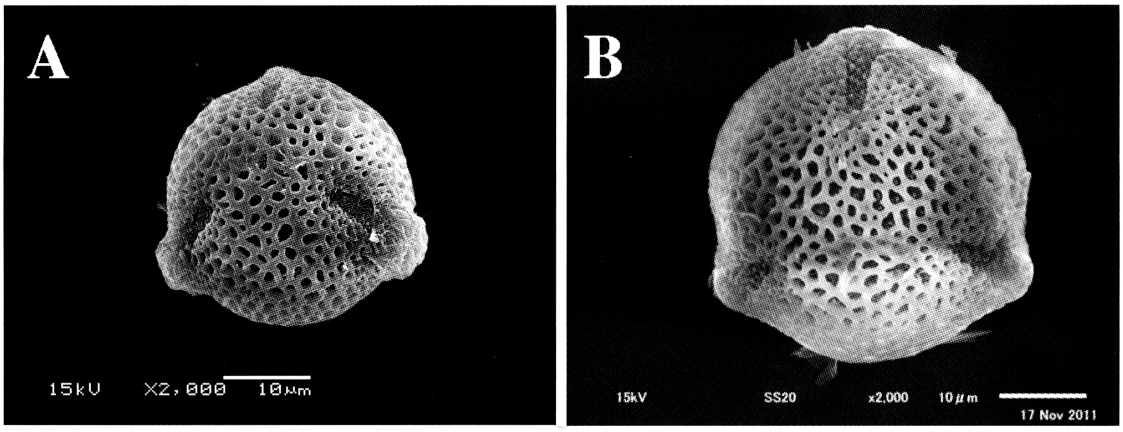

FIG. 5. Scanning electron micrographs of polar view of pollen grains from two floral morphs in the Akasaki population of *Psychotria serpens*. A, L-styled morph; B, S-styled morph. All scale bars represent 10 µm.

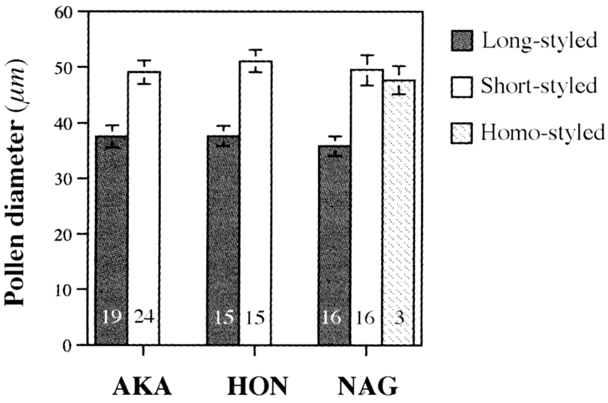

FIG. 6. Pollen diameter of L-, S-, and H-styled morphs in three populations of *Psychotria serpens*. Vertical lines indicate standard deviations. Numbers represent sample sizes. AKA, Akasaki population; HON, Honcha Pass population; NAG, Nago-dake population.

TABLE 3. Fruit set percentages under open pollination in the NAG population of *Psychotria serpens* on Okinawa Island. Values sharing the same letters (a, b) indicate no significant difference among floral morphs at $P < 0.01$ by Fisher's exact test corrected using the Bonferroni adjustment procedure.

Morph	No. of individuals examined	No. of inflorescences	No. of flowers examined	No. of flowers fruited	Fruit set %
S-styled	3	9	333	134	40.2 a
L-styled	5	12	277	148	53.4 b
H-styled	3	6	292	138	47.3 ab

found in three individuals, were identical in polar diameter with those from the S-styled morph in the same population (Table 2).

Fruit production under open pollination

Fruiting under open pollination occurred in the L- and S-styled morph flowers in all three populations examined. We quantified fruit set on three to five marked individuals with different flower morphs in the NAG population. Fruit set percentage of the L-styled morph flowers was significantly higher than in the S-styled morph flowers. The H-styled morph flowers also produced fruit, and the fruit set percentage did not differ significantly from that in the S-styled and L-styled morph flowers (Table 3).

Discussion

Our observations on floral variation in natural populations of *Psychotria serpens* found distylous individuals consisting of L- and S-styled morphs, as in other species of the genus (Hamilton 1989, Kondo *et al.* 2007, Sakai & Wright 2008), although these two morphs were not exactly reciprocal in stigma and anther heights. A short, H-styled morph occurred occasionally within a single population together with the L-

and S-styled morphs on Okinawa. The pollen grains of *P. serpens* were significantly larger in the S-styled morph than in the L-styled morph, as in other distylous species (Dulberger 1992, Kondo *et al.* 2007), and the pollen size ratio of S- to L-styled morphs (1.3-1.4 among the three study populations) were within the ranges reported previously for other distylous species (Dulberger 1992).

Stamens of individuals with the L- and S-styled morphs usually produced stainable pollen in all populations examined. In contrast, stamens of individuals with the H-styled morph lacked pollen grains or did not produce stainable pollen grains, suggesting that the stamens of this morph tend to be male sterile. Although the incompatibility system that prevents self- and intramorph mating is important for maintaining distyly, it was not examined in our study. In open pollination, flowers with the L-, S-, and H-styled morphs set fruit, although the fruit set percentages varied among the morphs. Our observations suggest that the L- and S-styled morphs are usually bisexual, whereas the H-styled morph frequently functions only as a female.

In the population of *Psychotria serpens* examined on Okinawa, flowers of the L-styled morph had statistically higher fruit set (53.4%) than those of the S-styled morph (40.2%). Similar patterns have been documented in other distylous species, and are usually interpreted as a reflection of more efficient pollen receiptivity by flowers of the L-styled morphs (Richards 1986). In *P. serpens*, the stigma of the L-styled morph flower is exserted from the corolla tube, whereas the stigma of the S-styled morph flower is concealed by obliquely oriented hairs found on the inner surface of the corolla tube. In accordance with differences in stigma position between the two morphs, the L-styled morph flowers appear to receive pollen grains more efficiently, although the pollination system for the species remains unclear.

As stated above, we found the short H-styled morph in some individuals of the NAG population on Okinawa. In distylous species, an H-styled morph has generally been viewed as resulting from recombination within the distylous supergene (Barrett 1992, Lewis & Jones 1992, Naiki 2012). The short H-styled morph in *Psychotria serpens* may be an example of such recombination. In flowers with the H-styled morph in the NAG population, however, the stamens lacked pollen or produced nonstainable pollen grains, suggesting abortion of the anthers. Furthermore, it was observed in a few H-styled morph flowers that one or two stamens were positioned above the stigma, like those of the S-styled morph flower, and contained stainable pollen similar in size to pollen of the S-styled morph flowers. Our observations suggest that *P. serpens* in the Ryukyu Islands is fundamentally distylous, like the species in Taiwan, and sometimes includes short H-styled morphs, stamens of which are frequently abortive. Considering this floral difference, the short H-styled morph in the NAG population appears to be a consequence of unusual flower development of the S-styled morph, although the factors underlying this unusual flower development are unknown.

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APPENDIX. 1. Pollen stainability of flowers in L-, S- and H-styled morphs in three populations of *Psychotria serpens*

Morph	No. of pollen grains examined	No. of stainable pollen grains	Pollen stainability (%)
Akasaki (AKA) pop.			
L-1	222	219	98.6
L-2	255	253	99.2
L-3	224	189	84.4
L-4	228	223	97.8
L-5	229	227	99.1
L-6	223	217	97.3
L-7	230	220	95.7
L-8	240	229	95.4
L-9	234	226	96.6
L-10	218	200	91.7
L-11	270	267	98.9
L-12	225	190	84.4
L-13	239	225	94.1
L-14	244	235	96.3
L-15	233	231	99.1
L-16	237	226	95.4
L-17	201	198	98.5
L-18	207	202	97.6
L-19	246	181	73.6
S-1	240	237	98.8
S-2	244	228	93.4
S-3	240	219	91.3
S-4	213	211	99.1
S-5	240	224	93.3
S-6	214	210	98.1
S-7	226	222	98.2
S-8	246	239	97.2
S-9	254	250	98.4
S-10	246	243	98.8
S-11	234	222	94.9
S-12	245	234	95.5
S-13	241	180	74.7
S-14	203	187	92.1
S-15	208	153	73.6
S-16	201	184	91.5
S-17	250	243	97.2
S-18	236	126	53.4
S-19	241	167	69.3
S-20	206	200	97.1
S-21	237	192	81.0
S-22	238	232	97.5
S-23	237	233	98.3
S-24	253	252	99.6
Honcha Pass (HON) pop.			
L-1	249	242	97.2
L-2	237	165	69.6
L-3	240	230	95.8
L-4	205	201	98.0
L-5	225	220	97.8
L-6	223	199	89.2
L-7	213	209	98.1
L-8	230	75	32.6
L-9	218	146	67.0
L-10	225	222	98.7
L-11	233	227	97.4
L-12	248	247	99.6
L-13	220	211	95.9
L-14	225	219	97.3
L-15	243	238	97.9
S-1	213	210	98.6
S-2	222	215	96.8
S-3	243	235	96.7
S-4	200	126	63.0
S-5	224	220	98.2
S-6	248	140	56.5
S-7	245	205	83.7
S-8	236	235	99.6
S-9	245	75	30.6
S-10	220	216	98.2
S-11	243	238	97.9
S-12	212	153	72.2
S-13	212	202	95.3
S-14	203	201	99.0
Nago-dake (NAG) pop.			
L-1	206	200	97.1
L-2	no pollen		
L-3	216	212	98.1
L-4	231	229	99.1
L-5	241	224	92.9
L-6	217	211	97.2
L-7	208	206	99.0
L-8	224	209	93.3
L-9	no pollen		
L-10	249	239	96.0
L-11	216	210	97.2
L-12	no pollen		
L-13	248	0	0.0
L-14	252	246	97.6
L-15	250	234	93.6
L-16	no pollen		
L-17	235	230	97.9
L-18	244	100	41.0
L-19	225	219	97.3
L-20	232	212	91.4
L-21	218	0	0.0
L-22	248	229	92.3
L-23	229	194	84.7
L-24	no pollen		
S-1	218	193	88.5
S-2	227	198	87.2
S-3	243	237	97.5
S-4	234	230	98.3
S-5	229	221	96.5
S-6	227	220	96.9
S-7	213	173	81.2
S-8	223	219	98.2
S-9	244	239	98.0
S-11	200	199	99.5
S-12	229	222	96.9
S-13	222	215	96.8
S-14	254	252	99.2
S-15	224	223	99.6
S-16	236	225	95.3
H-1	no pollen		
H-2	no pollen		
H-3	no pollen		
H-4	231	211	91.3
H-5	no pollen		
H-6	240	239	99.6
H-7	no pollen		
H-8	no pollen		
H-9	220	218	99.1